



NOTE

Wildlife Science

Record of *Cryptosporidium serpentis* from *Goniurosaurus kuroiwaie sengokui* (Reptilia, Squamata, Eublepharidae) in Tokashikijima, Okinawa Prefecture, Japan

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ABSTRACT. In reptiles, infection with *Cryptosporidium* parasite can be lethal, especially in species of family Eublepharidae such as *Eublepharis macularius* and *Goniurosaurus splendens* species. We investigated the presence of *Cryptosporidium* spp. in wild reptiles, especially *Goniurosaurus* spp., in five islands of Japan. Nested PCR was performed to detect *Cryptosporidium* spp. in 38 fecal samples of 9 reptile species from five islands of Japan, and 3 (3/8) positive samples of *Goniurosaurus kuroiwaie sengokui* from Tokashikijima, Okinawa Prefecture were detected. A BLAST search of the detected gene sequences revealed that the all 3 samples were identified as *Cryptosporidium serpentis*, a reptile-specific *Cryptosporidium* parasite. This is the first record of *Cryptosporidium* from a wild *Goniurosaurus* species in Japan and worldwide.

KEYWORDS: 18S rRNA, *Cryptosporidium*, *Goniurosaurus*, nested PCR, Okinawa Prefecture

Cryptosporidium spp. are protozoan parasites that are distributed worldwide with many vertebrates including reptiles as host [18]. *Cryptosporidium* parasites infect the gastrointestinal tract, mainly the stomach and intestines, and often cause severe symptoms such as diarrhea, vomiting and emaciation [3, 14, 18]. There is no effective treatment for the infection with parasites [12, 18]. *Cryptosporidium* is also known to be highly pathogenic not only to humans and domestic animals but also to pet reptiles. For example, it has been reported that the parasites caused gastritis in snakes, lizards and chameleons [2, 8, 11] and caused enteritis in tortoises, snakes and lizards [6, 12]. Infection with the parasites has also been reported to be lethal, especially in the leopard gecko *Eublepharis macularius* (Eublepharidae) [1, 17]. Experimental infection with *Goniurosaurus splendens*, a species of Eublepharidae in Japan, has also been reported to be lethal [5]. Therefore, if *Cryptosporidium* species were to become endemic in Japan, the impact on native Japanese species, especially *Goniurosaurus* species would be immeasurable.

There are four known *Cryptosporidium* to reptiles as host: *C. serpentis*, *C. varanii*, *C. testudines* and *C. ducismarci* [14]. *C. serpentis* is known to have different parasitic genotypes in snakes and lizards [18]. In Japan, there have been reports on the infection status in snakes and lizards in Honshu (from Aomori Prefecture to Yamaguchi Prefecture) and Kyushu (from Fukuoka Prefecture to Kagoshima Prefecture) [5, 9]. However, the current infection status in field reptiles in Japan, especially those in the Nansei Islands, has rarely been investigated.

In this study, we investigated *Cryptosporidium* infection in native reptiles, especially *Goniurosaurus* species, in the Nansei Islands. Two species with five subspecies of *Goniurosaurus* spp. inhabit the islands of Japan from Kagoshima Prefecture to Okinawa Prefecture. All species are designated as natural monuments of the prefecture in which they live and as Appendix III of the CITES. Recently, there are concerns about a decline in the number of reptiles due to habitat deterioration and other factors [7]. Therefore, investigation of *Cryptosporidium* infection in *Goniurosaurus* species in the regions is expected to provide important information from the perspective of conservation of endangered lizards.

In this study, 38 fecal samples were collected from nine species of reptiles (including those of *Goniurosaurus*) on five Islands in southern Japan (Amami-Oshima, Kikaijima, Tokashikijima, Miyakojima and Irabujima) in September 2020 (Table 1). When we found and temporarily captured reptiles, their feces were collected when they defecated. The collected feces were stored in sample tubes filled with anhydrous alcohol. The fecal samples of *Goniurosaurus* were obtained from a conservation project for *Goniurosaurus*

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Table 1. Detection of *Cryptosporidium* sp. in reptiles by region in this study

Species/Island	N	Tokashikijima	Amami-Oshima	Kikaijima	Miyakojima	Irabujima	No. of PCR positive/No. analyzed
<i>Gekko hokouensis</i>	13	3	3	1	1	5	0/13
<i>Gekko</i> sp.	1	1	-	-	-	-	0/1
<i>Hemidactylus frenatus</i>	2	-	-	-	2	-	0/2
<i>Goniurosaurus kuroiwae sengokui</i>	8	8	-	-	-	-	3/8
<i>Diploderma polygonatum polygonatum</i>	1	1	-	-	-	-	0/1
<i>Diploderma polygonatum ishigakiense</i>	6	-	-	-	4	2	0/6
<i>Scincella boettgeri</i>	1	-	-	-	-	1	0/1
<i>Takydromus smaragdinus</i>	5	-	-	5	-	-	0/5
<i>Lycodon semicarinatus</i>	1	-	1	-	-	-	0/1
Total	38	13	4	6	7	8	3/38

“-” means no fecal samples was collected.

carried out by the Ministry of the Environment, Japan. From the perspective of protection of endangered species, information on the location of samples will not be disclosed.

DNA from fecal samples was extracted using NucleoSpin Soil kit (Macherey-Nagel, Düren, Germany). As a pretreatment for DNA extraction, feces were crushed using Vortex Genie 2 Mixer (Scientific Industries, Bohemia, NY, USA) with MN Bead Tube Holder (Macherey-Nagel) as described in the instruction of the kit. Extracted DNA was stored at -25°C until further analysis.

Nested PCR to detect the 18S rRNA gene of reptile *Cryptosporidium* was performed using Tks Gflex DNA polymerase (TaKaRa Bio Inc., Kusatsu, Japan) with primers described previously [9]. Nested PCR produced approximately 850 bp sequence of 18S rRNA gene. The PCR products were subjected to 1% agarose gel electrophoresis, stained with ethidium bromide (Nacalai tesque, Kyoto, Japan), and then visualized under ultraviolet light. The DNA fragments obtained from PCR were excised from the gel and purified using the NucleoSpin Gel and PCR Cleanup kit (Macherey-Nagel). Nucleotide sequences of all of the PCR products were determined by the dideoxy chain termination method (FASMAC, Atsugi, Japan).

All sequences were aligned using the ClustalW option in MEGA 11 version 11.0.13 [16], and the alignment was later corrected by naked eye observation. The resultant new sequences were deposited in GenBank under accession numbers LC759647-759648. The respective accession numbers were registered with the name of the haplotype as Tokashiki 1 and Tokashiki 2. Phylogenetic analysis of closely related sequences was conducted to examine the phylogenetic status of the 18S rRNA associated with Tokashiki 1 and 2. The phylogenetic tree was reconstructed by the neighbor-joining method using MEGA 11 version 11.0.13 with the T92+G model selected as the best substitution model tested with MEGA 11 version 11.0.13 software [16]. Bootstrap analysis with 1,000 replicates was used to evaluate the reliability of the results of phylogenetic analysis [4].

Among the 38 fecal samples that were analyzed, 3 *Goniurosaurus kuroiwae sengokui* were positive for *Cryptosporidium* sp. (Table 1). All of the individuals in which *Cryptosporidium* was detected were adults (1 male and 2 females), and no emaciation was observed.

Gene sequencing and BLAST searches of the 3 samples resulted in all 3 samples matching the *Cryptosporidium serpentis* sequence (Tokashiki 1 matched 99.48% identical rate with accession number AY120915, Tokashiki 2 matched 99.87% identical rate with accession number MT626670). Seven nucleotide substitutions were observed in one of the 3 sequences. Furthermore, using the sequences obtained in this study and 16 sequences registered in GenBank as of February 2023, a phylogenetic tree with genotype names proposed by [18] was constructed (Fig. 1). *Cryptosporidium serpentis* found in this study formed a cluster with other *C. serpentis* (accession numbers MT626670, AY120915, AF151376).

C. serpentis detected in this study is known as a causative agent of gastritis in snakes and lizards [2, 8, 11]. *Cryptosporidium* infections have been confirmed in snakes (*Rhabdophis tigrinus*) in Honshu, but the species have not been identified [9]. However, in the BLAST analysis of the sequences (accession number: AB222185) detected in the paper [9], the identical rate is high for *C. parvum* (97.70% identical rate with accession number KT151545) or *C. baileyi* (97.70% identical rate with accession number KT151544). Therefore there is a large possibility that *Cryptosporidium* in snakes in Honshu is different from *C. serpentis* detected in this study. *C. serpentis* is a different species from *C. varanii*, for which infection in captive leopard geckos (*Eublepharis macularius*) has been confirmed overseas and in Japan [5]. However, the pathogenicity of *C. serpentis* for *Goniurosaurus* spp. is still unknown. Although *Cryptosporidium* infection has been confirmed in foreign captive individuals of *Goniurosaurus* sp. [13, 15], our cases are believed to be the first confirmed cases of infection from a field *Goniurosaurus* spp., either domestic or foreign. Waterborne *Cryptosporidium* infection has been reported [3, 14]. Since there are many streams and waterways of various sizes in Tokashikijima and since *Goniurosaurus* is terrestrial, it is possible that infection was transmitted through water. However, since *Cryptosporidium* is also a common parasite in soil [10], details of the infection route are unknown. The reason why parasite genes were not detected in other reptiles from which feces were collected in Tokashikijima may be that these reptiles are arboreal and have had little opportunity to engage with the groundwater system. Although other reptiles from which feces were collected on other islands are not only arboreal but also terrestrial, *Cryptosporidium* was not detected in their feces (Table 1). *Cryptosporidium* may be absent or rare on these islands, but the details are unknown and require further investigation.

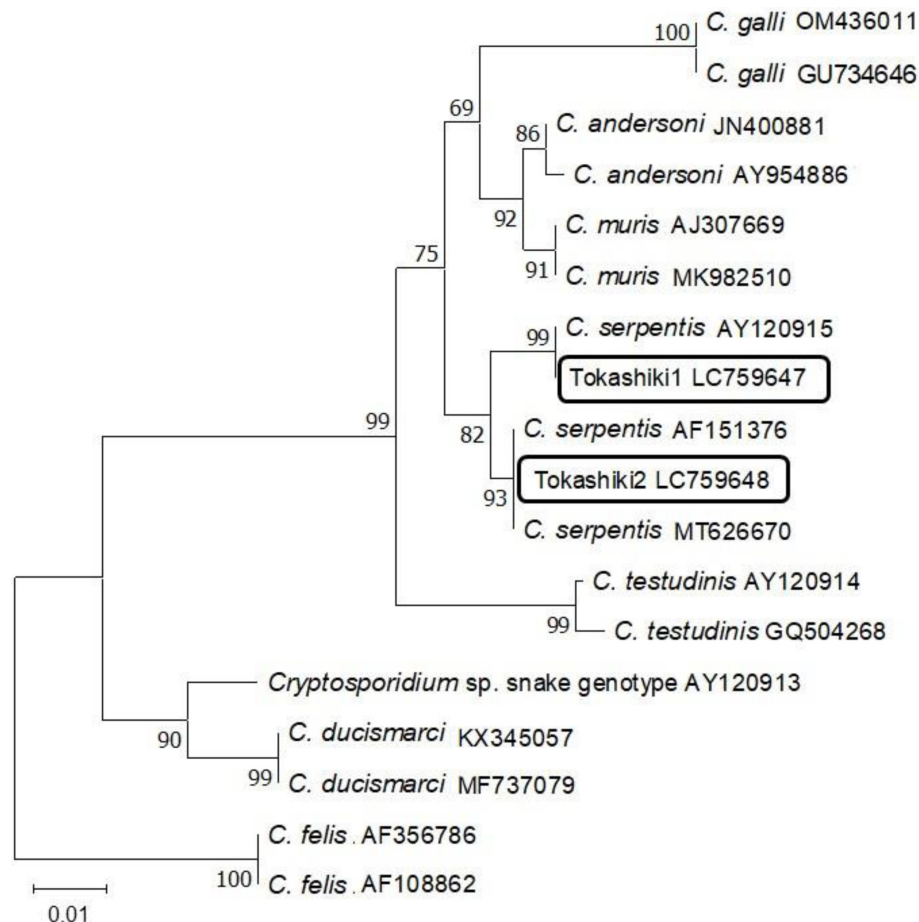


Fig. 1. Genetic relationship among named *Cryptosporidium* species and unnamed genotypes inferred by neighbor-joining analysis of the partial 18S rRNA gene. After excluding gaps and missing data, the sequence used was 415 bp. Values on branches are percent bootstrapping using 1,000 replicates. Numbers following species or genotypes are accession numbers. Sequences from this study are circled. *C. felis* (AF108862, AF356786) was used as an outgroup. The scale bar represents 0.01 nucleotide changes per position.

In conclusion, we detected for the first time reptile *Cryptosporidium* parasites, *C. serpentis*, from fecal samples of *G. kuroiwaie sengokui* naturally living in Tokashikijima, Okinawa Prefecture. The cases of infection detected in this study are believed to be the first confirmed cases of infection from *Goniurosaurus* spp. in the field, either domestically or internationally. Interestingly, *Cryptosporidium* was not detected in any other reptile or in any *Goniurosaurus* species in other islands. The pathogenicity of this protozoan in reptiles, the mode of transmission, and detailed geographic distribution remain unknown. However, the results of the present study will contribute to further investigations aimed at conservation of endangered species by monitoring the risk of population decline due to infectious diseases.

POTENTIAL CONFLICTS OF INTEREST. The authors have no conflicts of interest.

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